Nanosized Liposomes Containing Bile Salt: A Vesicular Nanocarrier for Enhancing Oral Bioavailability of BCS Class III Drug

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ABSTRACT - PURPOSE: Liposomes have been studied as a colloidal carrier in drug delivery systems, especially for oral administration. However, their low structural integrity in the gut is still a major shortcoming. Membrane disruptive effects of physiological bile salts in the small intestine result in premature drug release prior to intestinal absorption. Thus, we analyzed the stabilizing effect of sodium deoxycholate when incorporated into nano-sized liposomes. **METHOD:** Cefotaxime-loaded liposomes were prepared with different sodium deoxycholate concentrations (3.75- 30 mM) by rotary film evaporation followed by nano-size reduction. The physical integrity of liposomes was evaluated by monitoring cefotaxime leakage, particle sizes in different simulated physiological media. The oral bioavailability and pharmacokinetics of cefotaxime was assessed in rats (n = 6 per group) after single dose of drug-encapsulated in liposomes containing bile salt, drug in conventional liposomes, and cefotaxime solution (oral and intravenous). **RESULTS:** Simulated gastric fluid with low pH showed less effect on the stability of liposomes in comparison to media containing physiological bile salts. Liposomes containing 15 mM sodium deoxycholate were most stable in size and retained the majority of encapsulated cefotaxime even in fed state of simulated intestinal fluid being the most destructive media. Pharmacokinetics data showed an increase in C_{max} and AUC_{0-inf} in the following order: cefotaxime solution < conventional liposomes < liposomes made with bile salts. The total oral bioavailability of cefotaxime in liposomes containing bile salt was found to be 5-times higher compared to cefotaxime solution and twice as much as in conventional liposomes. **CONCLUSION:** Incorporation of bile salts, initially used as membrane permeation enhancer, also acted as a stabilizer against physiological bile salts. The nano-sized liposomes containing sodium deoxycholate were able to reduce the leakage of encapsulated cefotaxime in the gut due to the improved vesicle stability and to enhance the oral bioavailability of acid-labile drugs up to 5-fold.

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INTRODUCTION

To date, oral drug administration is the most popular and convenient route for drug administration. However, drug delivery via the gastrointestinal tract (GIT) faces several challenges still to be solved related to poor drug solubility, the poor permeability of the intestinal mucosal barrier or low stability toward the harsh conditions found in the GIT. While there is an increasing need for antibiotics in today´s society, many drug molecules have either limited intestinal permeability or are instable at gastric pH, therefore, they must be administered intravenously. In the present study, the third-generation cephalosporin cefotaxime (CEF) was chosen as model drug, because it is classified as a class III drug according to the Biopharmaceutical

Classification System (BCS) due to high solubility but low permeability (1). CEF is a semi-synthetic, 2-aminothiazolyl beta-lactam antibiotic that exhibits a high hydrophilic character. Its limited oral bioavailability can be attributed to rapid degradation as the β-lactam moiety undergoes hydrolysis at low pH found in the stomach (2). Furthermore, being a peptidomimetic drug, CEF exhibits a zwitterionic character that restricts the capability to permeate the intestinal mucosal membrane. The pharmacokinetic parameters after single injection have been presented in several species such as rats (3), sheep (4), goats (5), calves

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(6), cats (7), dogs (8), and humans (9). An approximate serum half-life of 1.1-1.3 h after injection was confirmed in different studies (10-13). The drug is primarily eliminated by the kidney, but also converted into the less active metabolite desacetyl-cefotaxime in the liver to a significant level (14-15). Considering the obvious drawbacks of drug administration by injection and potential side effects of high oral dosages, enhancing the oral bioavailability of CEF is a significant model study. Studies on oral bioavailability enhancement of BCS Class III cephalosporin antibiotics are limited in literature (16-19).

Liposomes have been widely studied as a colloidal carrier system to enhance the therapeutic efficacy of drugs (20-21). The amphiphilic character of phospholipids not only enhances the solubility of poorly soluble drugs by encapsulating them inside a lipid bilayer but also protects them from degradation, retards their leakage from vesicles and increases membrane penetration across the biological membrane (22). Through the past decades, various materials have been used to increase intestinal permeability such as chitosan and its derivatives (23), cyclodextrins (24), saponins (25), medium chain fatty acids (26), or bile salts (BS) (27). BS are naturally occurring amphipathic sterol surfactants. On the one hand, they enhance absorption by increasing drug solubility and the rate of dissolution or provide protection from degradation; on the other hand they are able to alter the membrane permeability through interaction with the membranes phospholipids (28). Yet, BS have been mainly applied as penetration enhancers in drug formulations to support mucosal absorption especially due to the membrane destabilizing properties (29-31). It was shown that BS significantly enhances the intestinal permeability of CEF and increasing the absorption rate up to 2-times (32). However, the harsh environment in the GIT has negative impact on the oral administration as the presence of physiological BS leads to solubilization of the lipid bilayer (33-34). The incorporation of BS enhancing the bioavailability of insulin colloidal drug carrier system has previously been reported (35-36). Thus, the integration of BS in the bilayer membrane might not only function as permeation enhancer, but also as a stabilizing agent to the vesicles preventing premature release of the drug in the harsh environment of the GIT.

To the best of our knowledge, this is the first time to combine nano-sized liposomes with BS to enhance oral bioavailability of CEF. The di-hydroxy BS sodium deoxycholate (NaDC) was chosen for this study as our previous study showed that its combination with mixed micelles has enhanced the oral bioavailability of CEF (37). However, the limitation of oral bioavailability improvement was limited by the drug hydrolysis in the acidic environment. Using a more stable vesicular system, the current study focused on the oral bioavailability of the Class III model drug CEF encapsulated in nano-sized liposomes incorporated with NaDC. After characterizations of nano-liposomes and verifying the vesicle's physical stability in various stimulated physiological media, the oral bioavailability and pharmacokinetics of CEF-loaded liposomes made with NaDC were compared with an aqueous drug solution and encapsulated drug in conventional liposomes using rats.

MATERIALS AND METHODS

Materials

Lecithin was acquired from Lipoid GmbH (Ludwigshafen, Germany). Cefotaxime sodium (CEF), sodium taurocholate (NaTC), sodium deoxycholate (NaDC), dihexadecyl phosphate, cholesterol, triton X-100 and pepsin were obtained from Sigma Aldrich (St. Louis, USA). Sodium heparin was acquired from CP Pharmaceutical Ltd, (Wrexham, UK) and Normal Saline Solution was purchased from Pfizer (NY, USA). Perchloric acid, glacial acetic acid, hydrochloric acid, a buffer of ammonium acetate, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium hydroxide, and the solvents acetonitrile, chloroform, tetrahydrofuran were obtained in high-performance liquid chromatography (HPLC) grade from Merck (Darmstadt, Germany).

Preparation of CEF-loaded nano-sized liposomes

The technique of thin-film hydration was applied to obtain CEF-loaded liposomes. Briefly, 200 µM of lipid mixture containing lecithin : cholesterol : dihexadecyl phosphate, (molar ratio 5:4:1) was dissolved in chloroform and a rotary evaporator (R-144Buchi, Switzerland) was used to build a thin lipid film by evaporating the solvent under reduced pressure for 20 min at 45ºC. In order to remove residual solvent, the flask was flushed with nitrogen. Thereafter, a 7 mL of CEF (21 mM) solution in phosphate buffered saline (PBS, 7.4 pH) were added to the flask together with glass beads. After the lipid film hydration, the CEF-

loaded liposomes were obtained by gently swirling the flask at 45ºC for 5 min. The vesicle suspension was exposed to 5 freeze-thaw cycles in liquid nitrogen. The vesicle size was reduced to nano-size by applying sonication at 150 W for 90 s using a prop-sonicator. Other series of CEF-loaded liposomes with BS were made in the same way but with incorporation of various BS concentrations (3.75, 7.5, 15, 30 mM) to CEF solution in PBS. Triplicate batches were prepared and kept in the refrigerator at 4ºC until needed. Samples were used within 12 h of preparation.

Preparation of drug solution

CEF solution (105 mM) in phosphate buffered saline(pH 7.4) for analysis was obtained by stirring at 37ºC for 10 min. Sterilized normal saline solution for injection was used to prepare the CEF solution for intravenous administration. Each solution was kept at -20ºC and used within 12 h of preparation.

Drug loading determination

A centrifugal filter device (Millipore Corporation, Bedford, USA) was used to centrifuge the CEF-loaded liposomes for 20 min at 15,000 rpm at room temperature. The HPLC method was used to immediately analyze the supernatant containing free drug after dilution. Triton-X 100 (1 %, w/w) was added in the ratio of 1:3 to release the loaded drug. Prior to analysis, the sample was centrifuged for 5 min at 12.000 rpm after vortexing for 45 s. The total drug loading was determined by equation 1.

Particle size and zeta potential measurements

Particle size, polydispersity index (PDI) and zeta potential (ZP) of nano-sized liposomes made with and without BS were determined using photon correlation spectroscopy (Malvern Zetasizer Nano ZS^{TM}, Malvern Instruments Ltd, UK). While, the size of vesicles before size reduction was measured using a Mastersizer-3000 laser diffraction particle size analyzer (Malvern Instruments Ltd. UK). The distribution of particle size was calculated as an average of three for each sample.

Scanning Electronic Microscopy (SEM)

Nano-sized liposomes made with and without BS were frozen then fractured and freeze-etched. Temperature was maintained at -180ºC. The samples were examined using a JSM-7610F field emission scanning electron microscope (JEOL, Tokyo, Japan) after the upper surface of samples was coated with gold-palladium alloy. SEM images of particles were taken using a high resolution camera fitted to the microscope.

Preparation of biorelevant incubation media

FaSSIF and FeSSIF were prepared as described in literature (38). The components of each medium are shown in Table 1. The compositions of SGF and SGF$_{pep}$ are also given in Table 1. The PBS solution was used as a control medium for this study.

$$\text{Drug Loading}(\%, \text{w/w}) = \frac{\text{Concentration of drug entrapped in the vesicles}}{\text{Total concentration of drug and lipids in the vesicles}} \times 100$$

Equation 1

Table 1. Compositions of various simulated physiological media used as incubation physiological media.

Ingredients	FeSSIF	FaSSIF	SGFpep	SGF
NaCl	11.87 g	6.19 g	2.0 g	2.0 g
Lecithin	3.75 mM	0.75 mM	-	-
10N NaOH	–	–	-	275 µL
NaOH pellets	4.04 g	0.35 g	-	-
Glacial acetic acid	144 mM	-	-	286 µL
NaTC	15 mM	3.75 mM	-	-
NaH$_2$PO$_4$	-	3.44 g	-	-
Pepsin	-	-	3.2 g	-
HCl conc.	-	-	7 mL	7 mL
Purified water qs.(mL)	1000 mL	1000 mL	1000 mL	1000 mL
pH	**5.0**	**6.5**	**1.2**	**1.2**

Evaluation of liposome integrity in various simulated media

Vesicles (0.5 mL) were transferred to 5 mL doubled-walled, temperature controlled beakers containing 4.5 mL of the incubation media. After the temperature was adjusted to 37ºC, the sample was added to the media and stirred at 100 rpm. Samples (150 µL) were collected at 60 min time.A control experiment was carried out using a CEF in PBS solution.

Total CEF was determined after adding 1% Triton-X 100 to the sample in a 1:3 ratio, followed by diluting with PBS and, finally, the addition of methanol in 1:1 ratio. Prior to HPLC analysis, the supernatant of the CEF sample was centrifuged for 5 min at 12000 rpm after vortexing for 45 s. Each sample was evaluated in triplicates and the values are reported as mean percentage of CEF remaining ± SD. The amount of remained drug for each simulated media was obtained by the following equation:

$$\text{Drug remaining (\%)} = 100 - \left[\left(\frac{CEF_i - CEF_0}{CEF_t - CEF_0} \right) \times 100 \right]$$

Equation 2

Where CEF_i is the amount of drug at different time points, CEF_0 is the amount of drug release in the initial CEF-loaded liposomes in PBS (i.e at t=0) and CEF_t is the amount of drug after destruction with 1% Triton.

Rat treatment

The animal experiment was approved by the Medical Faculty Novi Sad Animal Ethics Committee. 24 Male Wistar rats (age 2-3 months, weight 289 ± 5.1 g) were maintained under simulated natural habitat at 23 ± 1 °C, 12 h light/dark cycles, and given standard diet and water ad libitum. The rats were allowed to acclimatize before the pharmacokinetic studies for 48 h. Before treatment, the rats were fasted overnight until approximately 4 h post-dose. After dosing, the rates were individually housed in metabolism cages. During blood sampling, rats were kept warm and the cages were covered to provide protection and security and to minimize isolation stress. Immediately after the last blood sample, rats were sacrificed by CO_2 asphyxiation.

Pharmacokinetic study

The rats were randomly divided into 4 groups (n= 6, per group) as follows: 1- A single dose of CEF-loaded (60 mg/kg of CEF) liposomes in phosphate buffered saline containing 15 mM NaDC administered by oral gavage; 2- A single dose of CEF-loaded (60 mg/kg of CEF) conventional liposomes in phosphate buffered saline administered by oral gavage; 3- A single dose of CEF (60 mg/kg) solution dissolved in phosphate buffered saline administered by oral gavage; 4- A single dose of CEF (15 mg/kg) in sterilized normal saline solution for injection administered intravenously. For the oral studies in group 1 to 3, 100 µL blood samples were collectedfrom the tip of the tail into heparinized tubes at pre-dose and after 15, 30, 60, 90, 120, 150, 180, 240, 300, 420, 540, 720 min and 24 h after the dose. For group 4, samples were collected after 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 720 min and 24 h after dose. Each blood sample was replaced by an equal volume of saline buffer. The collected samples were immediately stored in the refrigerator at 4ºC and analyzed within 6 d.

HPLC determination of CEF

The CEF extraction from plasma samples was conducted following previously published methods (39-41). Briefly, an aliquot of plasma (100 µL) was transferred into Eppendorf micro-centrifuge tubes and deproteinized by adding 15 µL aliquots of 30% (w/w) perchloric acid. After vortex-mixing for 30 s, 70 µL of 0.55 M aqueous disodium hydrogen orthophosphate buffer was added and the mixture was immediately vortexed again for further 15 s. Afterwards, samples were centrifuged (Eppendorf, Hamburg, Germany) at 15,000 rpm for 10 min and 60 µL of the supernatant was injected into the HPLC system. Limit of quantification (LOQ) of this validated assay was 0.125 µg/mL and, at this concentration, intra- and inter-day coefficients of variance were 5.33 and 6.13 %, respectively. The recovery was > 95% over the concentration range tested (0.125 - 10 µg/mL). All CEF-plasma were analyzed by a Shimadzu's HPLC system (Shimadzu Corporation, Japan) consisting of a LC-10Avp Intelligent HPLC pump, a SPD-10Avp detector set at an operation wavelength of 254 nm, and a Rheodyne 7725i injector fitted with a 50 µL sample loop (Rheodyne, USA). A Phenomenex SB-C18 reversed phase analytical column (150 x 4.6 mm, 5gapµm) (Torrance, CA, USA) fitted with Refillable Phenomenex guard column (10 x 2 mm, 5 µm) was used with 0.04 M aqueous ammonium acetate: acetonitrile: THF (9:7:3, v/v) at pH 5.7 (adjusted using glacial acetic acid)as mobile phase at a flow rate of 0.9 mL/min. The obtained data were collected and processed by Shimadzu Class VP software. Data manipulation and processing

was carried out using EZChrom Elite software using peak area for quantification

Data analysis
Pharmacokinetic parameters, namely maximum concentration (C_{max}) and the time to reach maximum concentration (T_{max}), were directly obtained from the plotting of CEF serum concentration-versus time. The elimination rate constant (determined using the terminal three points) was used to calculate the half-life ($t_{1/2}$). The trapezoidal method was used to derive the area under the plasma concentration-time curve (AUC_{0-t}) and extrapolated to infinity to give $AUC_{0-\infty}$. The mean residence time (MRT) was determined from AUC_{0-t}. The oral bioavailability (F) was obtained by equation 3. All data were expressed as mean ± SD.

$$F\,(\%) \;=(\frac{AUC_{0-\infty\;oral}}{AUC_{0-\infty\;intravenous}} \times \frac{Dose_{intravenous}}{Dose_{oral}}) \times 100$$

Equation 3

STATISTICAL ANALYSIS

A statistical analysis was conducted using one-way analysis of variance to compare mean values of each variables considering a result statistically significant when $p<0.05$.

RESULTS

Characterizations of liposomes
An overview on vesicle size of liposomes made with various content of NaDC before size reduction are shown in Figure 1, whereas the sizes of similar vesicles after nano-size reduction and their polydispersity index (PDI), zeta potential (ZP), drug loading (DL) and their SEM images are shown in Table 2 and Figure 2, respectively. An increase in the vesicle sizes from 4.5 (± 0.9) to 11.4 (± 0.4) µm was achieved upon the increase of NaDC concentration up to 15mM. At higher NaDC concentration of 30 mM a drop in particle size to 3.4 (± 0.7) µm was detected. All liposomal formulations showed a fairly narrow size distribution. An analogous rise in drug loading was observed from 28.9 (± 2.7) to 46.3 (± 3.1) % increasing the BS concentration up to 15mM, followed by a reduction to 18.9 (± 2.7) % upon higher BS content. In addition, the ZP followed the same trend line slightly increasing in negativity from - 25.2 (± 3.5) to -28.8 (± 1.6) mV upon the increase in BS content to 15 mM followed by a decrease at higher concentration.

Stability evaluation of liposomes in various incubation media
To optimize the stability of nano-sized liposomes, the retaining amount of encapsulated CEF was evaluated in various simulated physiological media. Figure 3 shows the percentage of CEF retained by nano-sized liposomes prepared without or with 15 mM of NaDC incubated in physiological media containing SGF, SGF_{pep}, FaSSIF, FeSSIF and PBS at 37°C over a period of 4 h. Overall, the percentage of CEF retained in vesicles was significantly higher ($p<0.05$) in nano-sized liposomes made with BS in each incubation media. Furthermore, the leakage was significantly higher ($p<0.05$) in physiological sodium taurocholate (NaTC) containing media, such as FeSSIF and FaSSIF in comparison to the simulated gastrointestinal media SGF, SGF_{pep} at low pH (Figure 3). The percentage of CEF leakage was significantly higher ($p<0.05$) from conventional liposomes in comparison to nano-sized liposomes containing BS in FeSSIF and FaSSIF media over 4 h of incubation (Figure 3).

In the next step, the physical stability of nano-sized liposomes made with different contents of NaDC (up to 30 mM) was evaluated in FeSSIF simulated physiological media containing 15 mM of NaTC detected as the most destructive media (Figure 4). The percentage of CEF released from the nano-sized liposomes upon incubation at 37°C over 1 h is clearly dependent on the incorporated NaDC concentration in the lipid bilayer. The ability of the vesicles to retain > 80% of CEF was evident for nano-sized liposomes prepared with a BS content of 15 mM NaDC indicating their enhanced resistance to NaTC in the medium. A decrease in CEF leakage was detected after 1 h incubated in FeSSIF medium with increasing BS content: 0 mM NaDC (24.9 %) > 3.75 mM NaDC (13.6 %) > 7.5 mM NaDC (6.8 %) > 15 mM (3.9 %). Further increase of NaDC (30 mM) resulted in 17.6 % drug leakage almost as high as no incorporation of BS. As a result, the physical stability of nano-sized liposomes was at an optimum concentration of NaDC (15 mM).

Liposomal integrity
The physical integrity of nano-sized liposomes prepared with and without NaDC incubated in various physiological media containing SGF, SGF_{pep}, FaSSIF, FeSSIF and PBS at 37°C was measured after 4 h and 24 h (Figure 5 and 6). The average vesicle size was between 202.8 (± 12.7) and 209.5 (± 10.7) nm with a PDI ≤ 0.17 (± 0.04)

for liposomes with and without the inclusion of NaDC before incubation. A reduction in vesicles sizes for conventional liposomes (without BS) occurred after 4 h upon incubation in FaSSIF and FeSSIF media from 210.6 (± 4.3) nm to 153.3 (± 20.7) nm and 148.0 (± 15.3) nm, respectively, whereas no significant reduction occurred in the low pH physiological media (SGF and SGF$_{pep}$) comparable to PBS only. A further decrease in

vesicles sizes was detected after 24 h upon incubation to 113.6 (± 20.3) nm and 99.8 (± 12.4) nm. The size reduction after 24 in PBS stayed insignificant, while the particle size after 24 h incubated in SGF and SGF$_{pep}$ decreased to 137.0 (± 15.3) nm and 138.0 (± 16.5) nm. Overall, a more significant size reduction was observed in BS containing media in comparison to media with low pH.
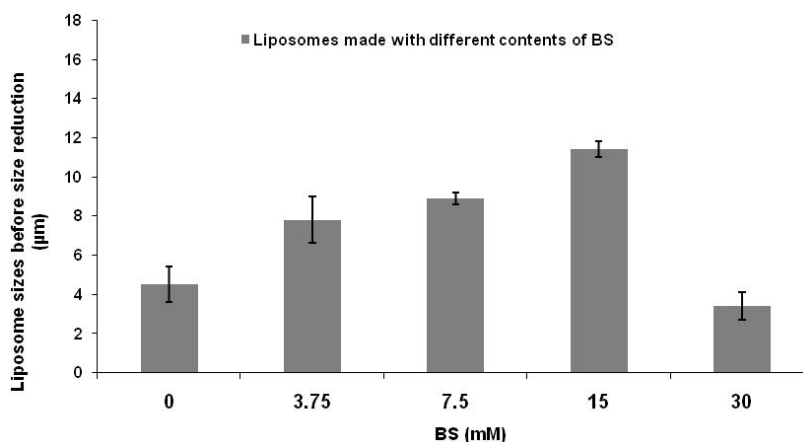

Figure 1: Vesicles sizes of CEF-loaded liposomes containing various contents of BS before sized reduction (data are mean ± S.D., n = 3).

Table 2. Nano-vesicles sizes, PDI, ZP and drug loading of CEF-loaded liposomes containing various contents of BS (data are mean ± S.D., n = 3).

BS contents (mM)	Vesicle size (nm)	Polydispersity Index (PDI)	Zeta potential (mV)	Drug loading (%)
0	199.0 ± 6.2	0.24 ± 0.01	- 25.2 ± 3.5	28.9 ± 2.7
3.75	163.8 ± 3.7	0.16 ± 0.02	-27.1 ± 6.4	29.6 ± 4.5
7.5	232.7 ± 8.8	0.27 ± 0.04	-27.5 ± 4.3	38.1 ± 2.6
15	197.2 ± 0.8	0.04 ± 0.02	-28.8 ± 1.6	46.3 ± 3.1
30	223.3 ± 38.5	0.18 ± 0.08	-25.5 ± 1.5	18.9 ± 2.7

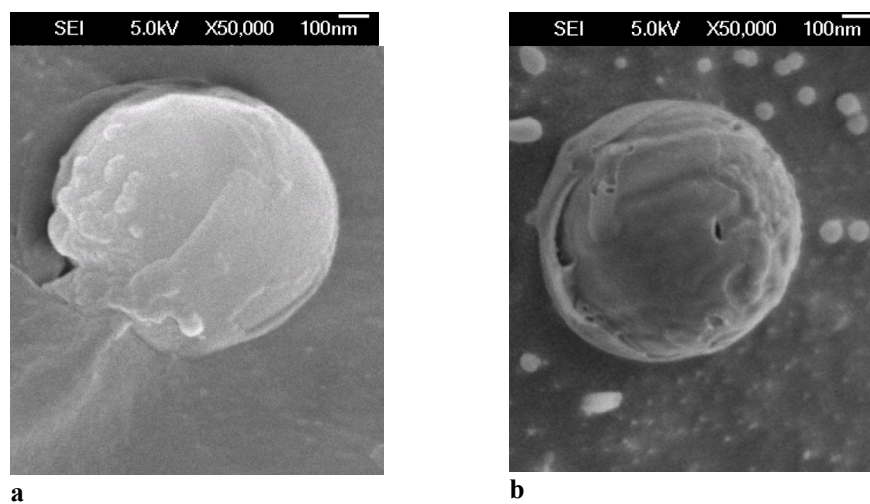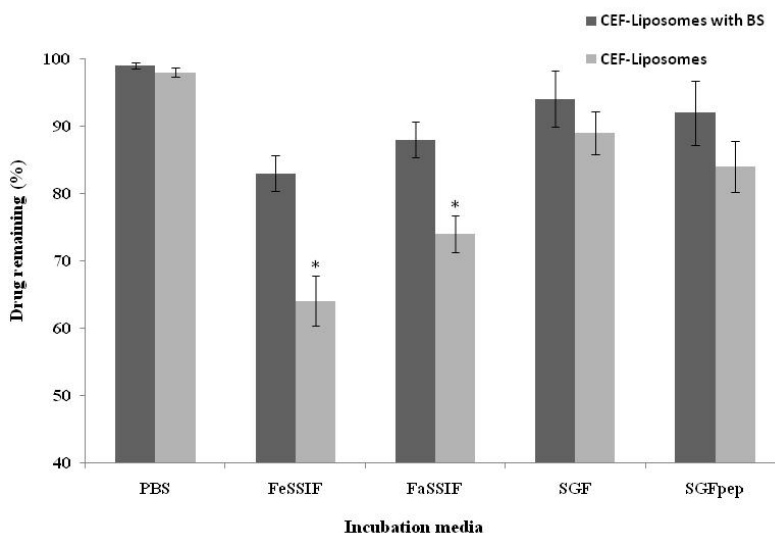a   b

Figure 2: SEM images of CEF-loaded Liposomes made without BS (a) and with 15 Mm NaDC (b). Caliper indicates 100 nm. Images were obtained by SEM under X 50000 magnifications operating at 5.0 kV.

* *p*<0.05 vs nano-sized liposomes made without BS

Figure 3: The percentage of CEF remaining in nano-sized liposomes made with and without BS after incubation at 37ºC over 4 h in various simulated physiological media (data are mean ± S.D., n = 3).
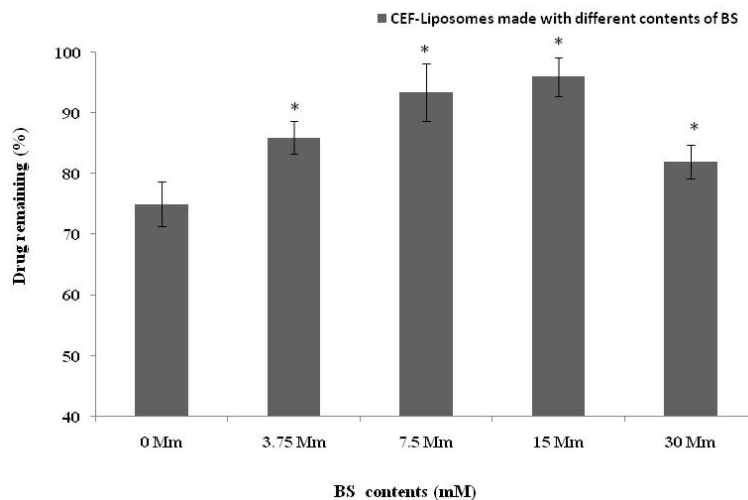
* *p*<0.05 vs nano-sized liposomesmade without BS

Figure 4: The percentage of CEF remaining in nano-sized liposomes made with different BS concentrations after incubation at 37ºC over 60 min in FeSSIF media contains 15 mMNaTC (data are mean ± S.D., n = 3).

Taking into account that nano-sized liposomes made with 15 mM of NaDC are at an optimum concentration of NaDC, this formulation was used in the next step to evaluate the physical integrity of the vesicles containing BS in the same physiological media as used before. In general, the vesicle size was comparably low affected even after 24 h of incubation even in FeSSIF detected as the most destructive media for nano-sized liposomes.

Pharmacokinetics evaluation of CEF-loaded liposomes in rats
Table 3 shows the values of the pharmacokinetic parameter, namely MRT, $AUC_{0-\infty}$, C_{max}, T_{max}, k_e, $t_{1/2}$ and F, achieved after oral administrations of CEF-loaded liposomes with and without BS, and from an aqueous solution of CEF after oral and intravenous administrations. The pharmacokinetic parameters for each formulation were analysed using a non-compartmental model. Overall, the

mean T_{max} value of the intravenous dosage was found to be 0.08 ± 0.1 h, whereas T_{max} value of all oral administered formulations, namely CEF-loaded liposomes with BS, was found to be $1.0 \pm$

0.0 h, CEF-loaded liposomes without BS was found to be 0.5 ± 0.0 h and CEF in aqueous solution was found to be 0.5 ± 0.0 h.
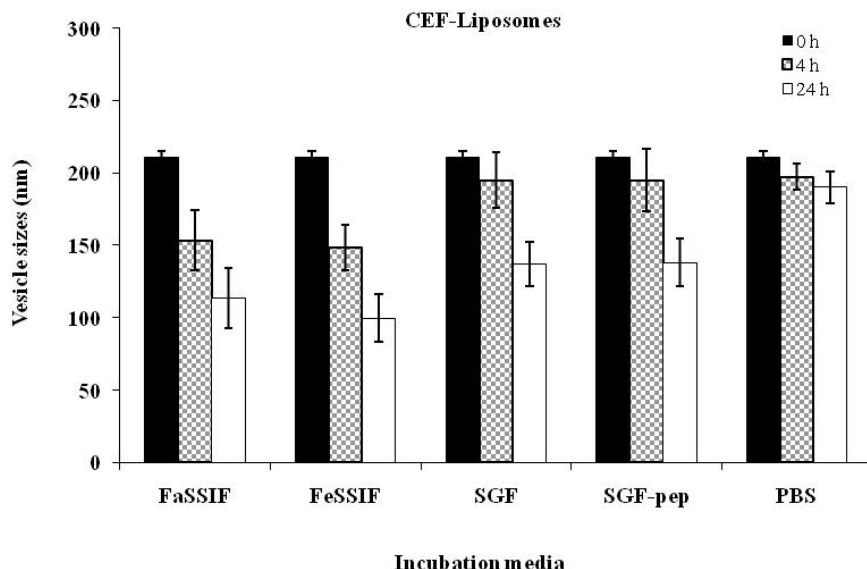

Figure 5: The vesicles size changes of nano-sized liposomes after incubation at 37ºC over 24 h in various simulated physiological media (data are mean $\pm$ S.D., n = 3).

Likewise, the mean C_{max} and $AUC_{0-\infty}$ values for the intravenous administration of CEF aqueous solution (50.4 ± 5.2 µg/mL and 20.4 ± 6.3 µg.h/mL) were detected to be comparably significantly higher (*p<0.05*). Nevertheless, comparing the mean C_{max} of all oral formulations, CEF in nano-sized liposomes with BS was superior (1.71 ± 0.3 µg/mL) to CEF in conventional

liposomes (1.29 ± 0.4 µg/mL) and CEF in aqueous solution (0.51 ± 0.2 µg/mL). Furthermore, the mean values of $AUC_{0-\infty}$ for CEF-loaded liposomes with BS were higher (5.73 ± 0.7 µg.h/mL) in comparison to conventional liposomes (2.87 ± 0.2 µg.h/mL) and CEF in aqueous solution (1.12 ± 0.5 µg.h/mL).
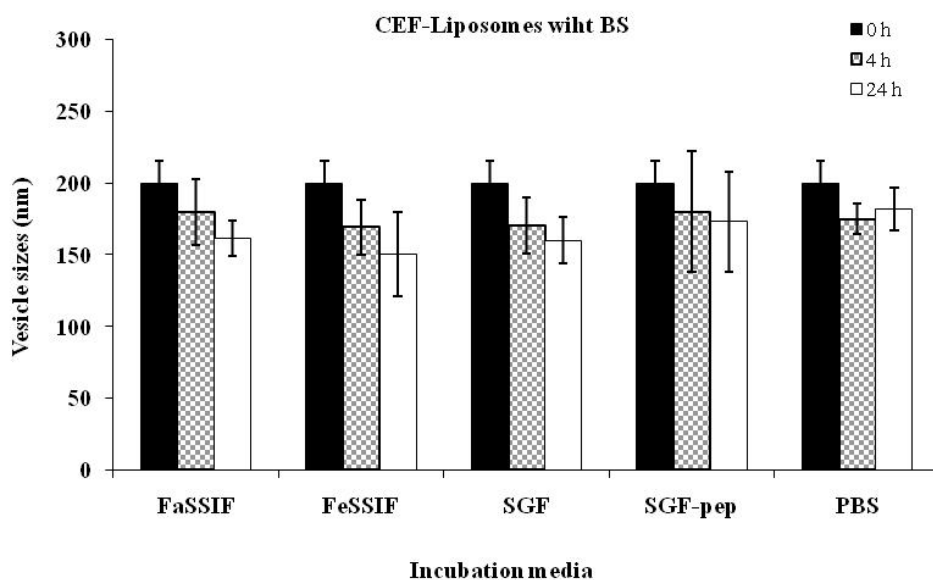

Figure 6: The vesicles size changes of nano-sized liposomes made with BS after incubation at 37ºC over 24 h in various simulated physiological media (data are mean $\pm$ S.D., n = 3).

Table 3. Pharmacokinetic parameters of CEF after intravenous injection of CEF solution, oral administration of CEF solution, CEF-loaded liposomes with and without BS (data are mean ± S.D, n = 6).

Parameters	CEF i.v.	CEF-PO	Lipo	Lipo-BS
C_{max} (µg/mL)	50.4± 5.2	0.51 ± 0.2	1.29 ± 0.4	1.71 ± 0.3*
T_{max} (h)	0.08 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	1.00 ± 0.0
MRT (h)	2.85 ± 0.2	4.57 ± 0.4	6.95 ± 0.8	8.29 ± 1.1*
$AUC_{0-\infty}$ (µg.h/mL)	20.4 ± 6.3	1.12 ± 0.5	2.87 ± 0.2	5.73 ± 0.7*
F (%)	-	1.37 ± 0.3	3.52 ± 0.6	7.02 ± 1.2*
k_e (h^{-1})	1.13 ± 0.4	0.50 ± 0.3	0.31 ± 0.1	0.23 ± 0.2*
$t_{1/2}$ (h)	0.60 ± 0.5	0.95 ± 0.6	1.50 ± 0.2*	2.10 ± 0.3*

Abbreviations: C_{max}: maximum concentration; T_{max} : time to reach maximum concentration; MRT: mean residence time;$AUC_{0-\infty}$: area under the plasma concentration-time to infinity; F: oral bioavailability; k_e: elimination rate constant.

* $p<0.05$ vs CEF-PO

Both parameters showed a relative wide inter-subject variation which might be related to different rat body weight and the resulting drug disposition. In total, the oral bioavailability after oral dosage was significantly higher ($p<0.05$) up to 2-fold incorporating CEF in nano-sized liposomes containing BS (7.02 ± 1.2 %) in comparison to conventional liposomes (3.52 ± 0.6 %), and 5.1-fold higher than administered as an aqueous solution (1.37 ± 0.3 %). The obtained values of the mean elimination rate constant (k_e), the mean resident time (MRT) and the elimination half-life ($t_{1/2}$) for each dosage form are presented in Table 3. The one-way ANOVA test showed significant differences ($p<0.05$) for k_e, and $t_{1/2}$ comparing CEF-loaded liposomes with and without BS and in aqueous solution. Figure 7 shows the mean plasma concentration level of CEF (60 mg/kg) after oral administration of the three dosage forms over a period of 6 h. Comparing the plasma concentration of CEF after intravenous and oral administration, it is obvious that CEF suffers from low oral bioavailability. However, it is obvious that the oral bioavailability significantly increased ($p<0.05$) using a liposomal formulation containing BS compared to conventional liposomesand CEF in aqueous solution.

DISCUSSION

In this study, we assumed that the inclusion of the NaDC into the vesicles could stabilize the lipid vesicle upon exposure to physiological BS in the GIT and enhance the oral bioavailability of the acid-labile CEF. Initial *in vitro* studies were conducted after vesicles formation were confirmed and characterized with the aim to evaluate the capacity of BS containing vesicles to withstand disruption in physiological BS solution. In order to achieve the optimum nano-sized liposome

formulation with NaDC, the effects of BS content (0, 3.75, 7.5, 15 and 30 mM) were evaluated on vesicle size before and after reduction, ZP, PDI, drug loading and leakage. The size of liposomes made with various NaDC contents increased with increasing the content in the vesicles up to a BS concentration of 15 mM. A significant reduction in vesicles sizes occurred after further increase of BS content in the vesicles. As described before, this phenomenon
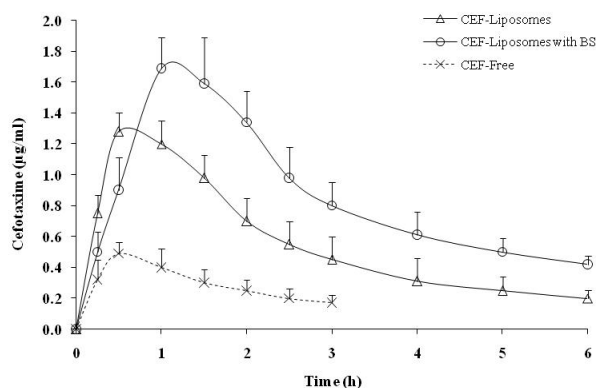

Figure 7: Plasma concentration-time profiles of CEF after oral administration of CEF (60 mg/kg) as free CEF in solution, CEF-loaded liposomes with and without BS given to rats (data are mean ± S.D., n = 6). **Abbreviations:** CEF: cefotaxime; BS: bile salt; CEF-Free: free cefotaxime in solution.

can be related to the BS-lipid bilayer-equilibrium which is interrupted above a certain BS concentration leading to solublize the lipid bilayer and reducing the formation of vesicle lamellas (42). The effect should be visible as a size decrease as seen for 30 mM of NaDC in the formulation. After nano-size reduction, the vesicles had an average size of 200 nm. A vesicle sizes as small as a few hundred nanometers was targeted detected to be a key in achieving good permeation across bio-

membranes (43). The amount of BS incorporation had no obvious impact on the mean PDI of the vesicles. The ZP is a common value to evaluate the stability of colloidal dispersions. All vesicles showed highly negative ZP values (-22.5 ± 3.5 to -28.8 ± 1.6 mV) as a result of the presence dihexadecyl phosphate with highly negative charge in the lipid composition, suggesting a dynamic stable system triggered by the electric repulsion between the particles (44). In general, the vesicular aggregation is hindered to flocculate if the ZP diverges from the iso-electric point (zeta potential = 0 mV). The value slightly increased rising the content of the negatively charged BS in the lipid bilayer up to 15 mM. At higher BS content the vesicles collapsed, as described before, which is confirmed by the drop in ZP.

The drug loading was observed to be linked to the particle size increasing with BS content. In addition, the gradual rise in entrapment efficiency might also be the result of enhanced viscosity of the micellar system supporting the stability of the internal aqueous phase and limiting the interaction with the outer aqueous media. The optimal nano-sized liposome formulation at 15 mM NaDC achieved a drug loading of CEF as high as 46.3 %. Afterwards, the amount of drug encapsulation in vesicles with 30 mM NaDC was compromised by the fact that higher concentration of BS reducing the lamellas of liposomes. However, the entrapment efficiency was lower than in conventional liposomes and therefore, a fluidizing effect of high BS in the lipid bilayers might lower the encapsulation capacity in addition.

With the aim to explore if the liposomal formulation might resist intestinal conditions, the physical stability of nano-sized liposomes with or without BS was examined by measuring the percentage of CEF remaining in the vesicles upon incubation in various simulated physiological media and PBS. Both vesicular systems showed almost no leakage in PBS as the permeability of CEF was hindered due to its anionic charge. Regarding the hypotonic media of SGF and SGF$_{pep}$, only 6 % and 8 % CEF leakage from nano-sized liposomes containing BS were detected over 4 h in SGF and SGF$_{pep}$, respectively. In comparison, further CEF leakage of 11 % and 19 % from conventional liposomes was detected over 4 h in SGF and SGF$_{pep}$, respectively. Facilitated permeation of CEF through the membrane was due to diffusion of protons from the media into the inner compartment compensation the ionic charge (45). Only minor change in vesicle size up to 4 h of incubation affirmed that the initial drug leakage is based on a proton gradient. Even if the drug remained inside the vesicle, a high chance of degradation should be considered based on its acid-induced degradability.

As seen in literature before, the effect of BS in the media on conventional liposomes was far greater than that of low pH (34). It was shown that a 10 mM BS solution resulted in over 80% release of the entrapped marker from the vesicle. Physiological BS concentrations in the GIT range from 1 to 20 mM (45). Thus, we tested the stability of our nano-vesicles formulations in FaSSIF and FeSSIF solution containing NaTC in a concentration of 3.75 and 15 mM, respectively. The leakage was obviously higher in FeSSIF in our study. As postulated before, physiological BS have a solubilzation effect on the liposomal membrane interacting with the phospholipids, and finally reducing the formation of vesicle lamellas upon increasing the concentration of BS above the CMC (NaTC ≥ 12 mM) (46-47). This effect was confirmed by decrease in vesicle size besides the continuous release of CEF.

A gradual increase of CEF leakage up to 12% and 18% were detected from nano-sized liposomes containing BS over 4 h in FaSSIF and FeSSIF media, respectively. Whereas, further increase of CEF leakage up to 26 % and 36% CEF leakage from conventional liposomes was determined over 4 h in FaSSIF and FeSSIF media, respectively. The greater loss of CEF in FeSSIF from conventional liposomes is presumably due to a disruptive effect of its higher NaTC concentration (15 mM). This greater stability of nano-sized liposomes containing BS made with higher NaDC concentrations compared to conventional liposomes clearly reflects increasing resistance to BS in the incubation medium. A similar release profile was observed in the literature investigating the stability of vesicles incubated in 10 mM NaDC (48). On the other hand, the size distribution, and therefore the membrane integrity, was measured after 4 h and 24 h of incubation to gain additional information on the mechanism in CEF leakage. The fact that the vesicle size was hardly affected for liposomes with BS, it was in accordance with the concept of transient and fast-resealing transient membrane pores upon exposure to BS in media (42). A major reason for the increased stability might be due to structural changes in the vesicles. As seen in literature, the incorporation of BS made the lipid membrane more deformable (49-50) which might alter the ion permeation. Changing the membrane viscosity might also change the interaction and uptake of physiological BS. Moreover, vesicles containing BS might be more stable due to an increased negative charge

preventing the transition into micelles as a result of anionically repulsion (51). In addition, BS were detected to protect from proteolytic degradation (52-53).

Furthermore, the critical BS content required for a maximum protective effect was evaluated. Raising the amount of NaDC in liposomes from 0 mM to 15 mM the CEF leakage reduced. An explanation for this observation might be a reduced diffusion potential originated by a decreased concentration gradient. With a concentration of 30 mM BS in liposomes, the drug leakage was greater than that from liposomes without BS upon contact with sodium taurocholate in media. This might be attributed to a change in rigidity and packing order of lipids especially when more or less than 15 mM NaDC was incorporated in their bilayers. The protective characteristics of NaDC inside liposomes contribute to improve oral bioavailability of CEF as more intact vesicles arrive at the absorption sites.

The oral bioavailability of CEF was evaluated on the basis of the plasma level. The pharmacokinetic studies showed that the PK of CEF encapsulated in liposomes made with BS was superior to the aqueous solution of CEF and conventional liposomes resulting in a higher $AUC_{0-\infty}$, C_{max}, T_{max}, $t_{1/2}$ and F. Although at this stage the ultimate fate and the mechanisms of absorption of liposomes with NaDC after oral administration have not been completely determined yet, the enhancement must be the result of a combination of positive effects before, on and through the intestinal epithelial membrane. Starting with better protection against hydrolytic degradation in the GIT environment, the prolonged life time increased the number of vesicles arriving in the intestine (54). Earlier results in literature showed that increased oral bioavailability was supported by the accumulation of vesicles at the brush-boarder membrane of enterocytes followed by an improved drug transport through the intestinal membrane facilitating the absorption into the systemic circulation (54). Drug formulations can be absorbed by biomembranes through multiple passive or active pathways using paracellular or transcellular transport. Herein, physicochemical parameters are the controlling factors, such as size, surface, lipophilicity, hydrophilicity, or polarity. Increased bioavailability using BS was explained by enhanced vesicle uptake and transport through the intestinal membrane (55), or the vesicle absorption by enterocytes through endocytosis (56-57). BS were also detected to increase the paracellular permeation of CEF by a mucolytic effect and opening the tight junctions by binding to calcium ions (58). In addition, BS were able to rise the attraction for transporters like H+/peptide symporter (PEPTl) and enhance the beta-lactam antibiotic trans-epithelial transportation and availability (59-60). Generally, the enhanced membrane permeation seems to be the sum of multiple mechanisms triggered by BS-loaded nano-sized liposomes.

Within the GIT tract the fate of lipid based vesicles, and therefore the drug release order, might be strongly influenced by enzymes such as lipases and phospholipases. Early vesicle hydrolysis can be triggered by gastric lipase (acidic lipase) and pancreatic lipase (alkaline lipase) in the stomach and the intestine. In this study, it is assumed that CEF was released from the lipid based vesicles prior to intestinal absorption. Phospholipase A_2 (PLA_2) was detected to regulate the digestion of phospholipids in the small intestine by catalyzing the hydrolysis of the ester linkage of the sn-2 position (second carbons of the glycerol molecule in the phospholipids) of the vesicle phospholipids lecithin in the presence of calcium ions producing the lyso-phospholipid hydrolyzed lecithin and the fatty acid (61-62). As a result, vesicle membrane permeability would quickly increase accompanied by the loss of loaded drug contents. This mechanism is supported by literature, as PLA_2 was detected to be highly active on the interface of well- organized nano-sized substrates, such as micelles and lipid bilayers (63-64). Under acidic conditions in the stomach the free drug is sensitive to hydrolysis. Chemical modification and pro-drug approaches can enhance the bioavailability of poorly permeable drugs (65). In this study, the enhanced CEF concentration in the blood stream as compared to the free drug was due to the protective encapsulation of the drug into liposomes. The BS containing CEF liposomes retain and protect the drug better as compared to the CEF liposomes. This explains the *in vivo* slower absorption and increase in AUC compared to the CEF liposomes.

CONCLUSION

In order to enhance oral bioavailability of BCS class III drugs, the physical stability of vesicles highly depends on their integrity in the GIT. The current study demonstrated that the incorporation of NaDC into nano-sized liposomes enhanced oral bioavailability of CEF up to 5-times. The liposomal formulation prepared at optimum incorporated 15 mM of NaDC in the lipid bilayer and revealed an entrapment efficiency of 46.3 % with a particle size of 197.2 ± 0.8 nm.

Conventional liposomes showed a gradual leakage of CEF in biorelevant dissolution media. In comparison, embedded NaDC in nano-sized liposomes protected the lipid bilayers of vesicles from leakage up to some degree suppressing vesicle disturbance and micellar solubilization effect. *In vitro* experiments suggested that nano-sized liposomes with NaDC had a protective effect during the transit through the GIT. Change in physical structural properties of vesicles, such as additional elasticity, fluidity and negative charge particles, might be the key factor for enhanced stability and intestinal permeability. In conclusion, the integration of BS as a part in lipid-based vesicular systems might represent a promising approach to improve oral bioavailability of BCS class III drugs.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to disclose.

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